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A mechanistic explanation for pH-dependent ambient aquatic toxicity of *Prymnesium parvum* carter

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ABSTRACT

The harmful algal bloom species *Prymnesium parvum* has caused millions of dollars in damage to fisheries around the world. These fish kills have been attributed to *P. parvum* releasing a mixture of toxins in the water. The characterized toxins, reported as prymnesin-1 and -2, have structural similarities consistent with other known ionizable compounds (e.g., ammonia). We investigated whether pH affects the toxicity of *P. parvum* under conditions representative of inland Texas reservoirs experiencing ambient toxicity from bloom formation. We evaluated pH influences on toxicity in laboratory and field samples, and modeled the physicochemical properties of prymnesins. Aquatic toxicity to a model fish and cladoceran was reduced by lowering pH in samples obtained from reservoirs experiencing *P. parvum* blooms; similar observations were confirmed for experiments with laboratory cultures. A pKa value of 8.9 was predicted for the prymnesins, which suggests that ionization states of these toxins may change appreciably over surface water pH of inland waters. These findings indicate that ionization states of toxins released by *P. parvum* may strongly influence site-specific toxicity and subsequent impacts to fisheries. Consequently, these results emphasize the importance of understanding processes that affect pH during *P. parvum* blooms, which may improve predictions of ambient toxicity.

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1. Introduction

Harmful algal blooms (HABs) may have devastating impacts on aquatic ecosystems, resulting in severe impacts to fisheries. Increases in the frequency and severity of HABs on the global scale has triggered scientific inquiry to define

factors causing these trends (Zingone and Enevoldsen, 2000; Anderson et al., 2002; Hallegraeff, 2003); however, among the greatest challenges for managers is the spread of invasive species. *Prymnesium parvum* is an example of an invasive HAB species that has transitioned from marine origins to inland systems. Identified nearly a century ago as a problem in marine environments because of its toxic blooms (Liebert and Deerns, 1920), *P. parvum* is more recently recognized as an invasive species threatening inland systems in the arid and semiarid southwestern and south central United States (Baker et al., 2007; Roelke et al., 2007; Schwierzke et al., in press).

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Anthropogenic changes to the hydrologic cycle, eutrophication, and salinization of waterways are associated with the spread of HABs (Anderson et al., 2002). Such changes, intertwined with climatological and geological factors, have rendered some Texas reservoirs to be within the tolerance range of *P. parvum* (Larsen and Bryant, 1998; Baker et al., 2007; Baker et al., 2009). Specifically, the species' euryhaline nature has apparently facilitated its transition from coastal marine and estuarine ecosystems to these weakly saline inland impoundments. Since the first harmful blooms of *P. parvum* in Texas were documented in the Pecos River (James and De La Cruz, 1989), *P. parvum* has spread to other systems in Texas resulting in toxic blooms and fish kills (Roelke et al., 2007; Schwierzke et al., in press).

P. parvum is a mixotrophic haptophyte that can gain energy photosynthetically as well as phagotrophically by feeding on other microorganisms (Skovgaard and Hansen, 2003). Exposure to *P. parvum* toxins can lyse cells (Yariv and Hestrin, 1961; Tillmann, 2003), disrupt cell membrane integrity (Yariv and Hestrin, 1961; Padilla, 1970; Kim and Padilla, 1977; Brooks et al., in press), and affect gill functions of aquatic organisms (Ulitzur and Shilo, 1966). Ecologists have proposed several purposes for the production and release of toxins by *P. parvum*, including acquisition of prey (Stoecker et al., 2006), elimination of algal competitors (Fistarol et al., 2003; Granéli and Hansen, 2006; Uronen et al., 2007), or reduced grazing pressure (Rosetta and McManus, 2003; Tillmann, 2003). A variety of factors, including nutrient limitation, salinity, temperature, and light are known to influence cell growth and the toxicity of laboratory cultures of *P. parvum* (Shilo and Aschner, 1953; Padilla, 1970; Dafni and Shilo, 1966; Larsen et al., 1993; Larsen and Bryant, 1998; Johansson and Granéli, 1999; Granéli and Johansson, 2003; Baker et al., 2007; Baker et al., 2009). Few studies have focused on factors governing the behavior of the toxins once they are released, or considered how bloom formation might alter the environment (e.g., light attenuation, nutrient availability, dissolved oxygen, pH) in ways that could influence the bioavailability and potency of *P. parvum* toxins.

Shilo and Aschner (1953) proposed that *P. parvum* toxins were proteins with high molecular weights. To date, the only characterized toxins are prymnesin-1 and -2, large chains of 90 carbon atoms and trans-1,6-dioxadecaline units with conjugated double/triple bonds at each terminal end; their respective chemical formulas are $C_{107}H_{154}Cl_3NO_{44}$ and $C_{96}H_{136}Cl_3NO_{35}$ (Igarashi et al., 1999). These compounds are amphiphilic, with uneven distributions of sugars and hydroxyl groups, and three chlorine atoms and one nitrogen atom. Both prymnesins are structurally similar to other HAB toxins such as maitotoxin and ciguatoxin, which are characterized by a network of hydroxylated polycyclic ether units (Murata and Yasumoto, 2000). The amine present on the prymnesins suggests that these compounds might be weak ionizable bases with pKa values >8. Prior studies suggested that some of the toxins released by *P. parvum* are ionizable, becoming more toxic to fish exposed at higher pH, with toxicity eliminated below pH 7 (Shilo and Ashner, 1953; McLaughlin, 1958; Ulitzur and Shilo, 1964).

However, these experiments were completed under marine conditions, and prior to the development of standardized aquatic bioassays.

This study examines whether pH also influences the toxicity of *P. parvum* toxin in less saline waters representative of Texas reservoirs where blooms have occurred. Simultaneous bioassays were performed at three pH levels with samples obtained during *P. parvum* blooms occurring in 2007 from two reservoirs, and with samples of laboratory cultures and culture filtrates. Further, the chemical structures of prymnesin-1 and -2 were examined to estimate their physicochemical properties. We hypothesized that toxins released by *P. parvum* are ionizable weak bases.

2. Material and methods

2.1. Bioassays with samples obtained from reservoirs experiencing blooms

2.1.1. Lake Whitney

Lake Whitney is a reservoir constructed in 1951 on the Brazos River, with a capacity of $4.68 \times 10^8 \text{ m}^3$, surface area of 95 km^2 , and shoreline of 362 km (Bailes and Hudson, 1982). Two 4-L samples were collected in NALGENE® I-Chem Certified Series™ 300 LDPE Cubitainers™ (Fisher Scientific) from Lake Whitney during a bloom in March 2007, transported to the laboratory on ice, and stored under refrigeration at 4°C . This lake sample contained 61.5×10^3 *P. parvum* cells mL^{-1} (enumerated microscopically with a hemocytometer). Ambient pH at the site in Lake Whitney when the sample was collected was pH 8.4. Total ammonia in the samples was <1 mg/L in whole samples, which is below ambient water quality criteria for the temperature and pH at which our experiments were completed. Dilutions in our toxicity experiments at which toxicity was observed further confirmed that dose dependent responses were not due to ammonia. Toxicity tests were initiated within 96 h of sample collection following EPA recommendations for ambient toxicity studies (US EPA, 2002).

Acute bioassays with <48 h old *Pimephales promelas* were conducted in 100-mL glass beakers. Three replicates of seven individuals were prepared for each treatment level. Reconstituted hard water (RHW) prepared according to APHA et al. (1998) was used as the diluent and control (treatment consisting of 100% RHW). Treatment levels were prepared by diluting lake water with RHW to the following percentages of lake water: control (RHW), 0.01, 0.1, 1, 5, 10, and 20%. These treatment levels were selected based upon ambient toxicity data from prior water quality monitoring efforts. A volume of 3-L was prepared for each treatment level, which was then divided into three aliquots of 1-L that were then adjusted to pH units of 6.5, 7.5, or 8.5 (+0.05) prior to dispensing experimental aliquots. The pH adjustments were achieved by slowly titrating 10% HPLC-grade nitric acid, which generally followed U.S. Environmental Protection Agency protocols for pH adjustment in Toxicity Identification Evaluations (US EPA, 1991). Test individuals were fed newly hatched brine shrimp (*Artemia* sp.) 2 h prior to the exposure, but were not fed during experiments (US EPA, 2002). Survivorship

was assessed at 24 and 48 h, and temperature, dissolved oxygen, and pH were measured at test initiation and completion. Exposures were conducted at 25 ± 1 °C under a 16:8 light:dark photoperiod.

A 10 d *Daphnia magna* reproductive study was also completed with Lake Whitney water (US EPA, 1994, modified as in Dzialowski et al., 2006). Treatment levels consisted of control (RHW), 12, 25, 50, and 100% lake sample water. Test solutions were prepared and adjusted to desired pH as previously described. Experimental units were 100-mL beakers filled with 80 mL of test solution. Organisms were fed daily with *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) augmented with filtrate from a cerophyll suspension (approximately 2 g/L RHW). The final green algae cell concentration was 30×10^6 cells mL⁻¹. One *D. magna* individual <24 h old was introduced in each beaker and transferred every other day to fresh test solution. Five replicates were prepared for each treatment level. Survivorship and fecundity were monitored daily. The experiment was completed for 10 d at 25 ± 1 °C under a 16:8 light:dark photoperiod.

2.1.2. Lake Granbury

Lake Granbury is a reservoir constructed in 1969, with a capacity of 167×10^6 m³, a surface area of 34 km², and an average depth of ~5 meters. Samples were collected during a *P. parvum* bloom that caused fish kills in March 2007 at three fixed monitoring stations and were handled prior to bioassay initiation as previously described. Ambient pH at the sites while the samples were collected ranged between pH 8.2 and 8.4. Total ammonia concentrations were <0.25 mg/l in the three samples, which again were lower than levels associated with ammonia toxicity (US EPA, 1999). Cell counts for samples from Sites 1–3 were 29×10^3 , 36×10^3 , and 36×10^3 cells mL⁻¹, respectively.

Experiments were conducted with *P. promelas* similar to those previously described in order to assess acute toxicity at the three sites. Six treatments, including a control (RHW), 6, 12, 25, 50, and 100% lake water were prepared using RHW as the diluent and adjusted to pH 6.5, 7.5 and 8.5. A 96-h acute exposure experiment was initiated with <24 h old *D. magna* using a composite sample from the three stations. Treatments included a control (RHW), 6, 12, 25, 50, and 100% lake water. Ten replicates were prepared for each treatment level. Exposures were completed in 100-mL glass beakers filled with 80 mL of test solution, and water in experimental units were renewed at 48 h. Organisms were fed daily the same concentration of the mixture described for the 10 d *D. magna* experiment, at which time survivorship was assessed. Exposures were conducted at 25 ± 1 °C under a 16:8 light:dark photoperiod.

2.2. Laboratory culture preparation

The UTEX LL 2797 (University of Texas, Austin, Texas, USA) strain of *P. parvum* was used to initiate cultures. Cultures were grown in 20-L glass carboys filled with 14-L of an artificial seawater (ASW) prepared according to Berges et al. 2001 and then diluted to a working salinity of 5.8 g L⁻¹ with ultrapure water (18 MΩ cm⁻¹). Afterwards, nutrients (NaNO₃ and NaH₂PO₄) were added at

concentrations of f/2 and f/8 media (Guillard, 1975); vitamins and trace metals were the same for both types of media. Three replicates were prepared for each treatment and all carboys were inoculated with 10^3 cells mL⁻¹ of *P. parvum* from stock cultures in late exponential phase grown in the corresponding medium at the stock salinity of 5.8 g L⁻¹. Cultures were maintained in incubators at 20 ± 1 °C for a 12:12 light:dark cycle with an irradiance of ~140 μE m⁻² d⁻¹. Carboys were repositioned and mixed daily by gently swirling.

2.3. Bioassays with samples obtained from laboratory cultures

Several experiments with larval *P. promelas* were completed using these *P. parvum* cultures. An initial experiment examined the toxicity of the three replicate carboys of both the f/2 and f/8 cultures. ASW adjusted to a salinity of 5.8 g L⁻¹ used in the media served as the diluent and controls. Bioassays were completed in 100-ml beakers filled to capacity with test solution. Treatments included a control (ASW), 1, 2.5, 10, 25, and 100% culture water containing *P. parvum* cells. Four replicates of five individuals were prepared for each treatment. Experiments were conducted at 25 ± 1 °C under a 16:8 light:dark photoperiod.

For subsequent studies, we separately pooled f/2 and f/8 cultures and then filtered half of each volume through GF/C filters (Whatman GF/C; VWR International, West Chester, Pennsylvania, USA). Acute toxicity to *P. promelas* was determined for cultures (f/2, f/8) that were unfiltered and filtered (cell-free filtrate), then these samples were manipulated to pH 6.5, 7.5 or 8.5 following procedures previously outlined. ASW adjusted to a salinity of 5.8 g L⁻¹ was used as the diluent and control. An additional RHW treatment for quality assurance was also prepared. Each acute toxicity study included a control (ASW), RHW, 0.1, 1, 5, 10, 25, and 100% media treatment. Four replicates of five *P. promelas* <48 h old were used for each treatment level. Experiments were conducted at 25 ± 1 °C under a 16:8 light:dark photoperiod.

2.4. Statistical analysis

LC₅₀ values for acute toxicity to *P. promelas* and *D. magna* were calculated by Probit analysis if data met assumptions; otherwise, the Trimmed Spearman-Kärber method was applied using TOXSTAT computer software (US EPA 2002). SAS (SAS Institute, Cary, NC, USA) was used for other statistical analyses. For the 10 d experiment with *D. magna*, significant differences in survivorship between the control at each of the respective pH treatments and reservoir water dilutions were determined using Fisher's Exact Test. Significant differences in reproduction were assessed by an ANOVA comparing the mean neonate production per female for all treatments ($\alpha = 0.05$), followed by Dunnett's test comparing the controls to each of the treatments at a respective pH ($\alpha = 0.05$). In addition, we compared the mean control responses between different pH levels for the respective endpoints using ANOVAs for each series of experiments to confirm health of test organisms.

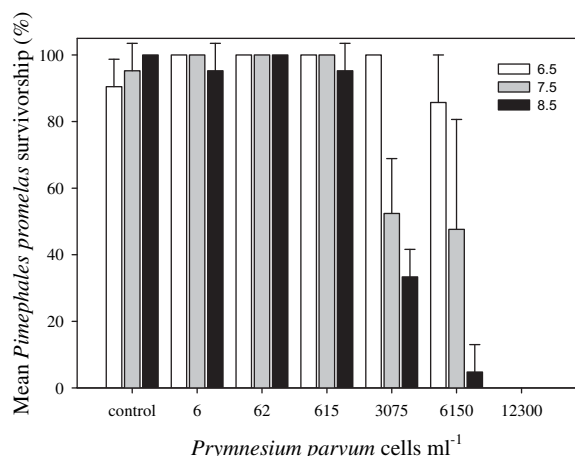


Fig. 1. Mean survivorship (\pm SD, $n = 3$) of *Pimephales promelas* exposed to dilutions of Lake Whitney water collected during a bloom of *Prymnesium parvum* in 2007. Cell density is expressed as the % lake water multiplied by the density of *Prymnesium parvum* cells in the undiluted sample. The error bars represent the standard deviation. Missing error bars are due to 100% survivorship in all replicates for a treatments, hence there was no variation to derive a prediction.

2.5. Estimation of prymnesin-1 and -2 physicochemical properties

Calculation of physicochemical parameters for prymnesin-1 and -2 (Fig. 1) was carried out using ACD/Labs (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada) ChemSketch, pKa calculator, LogD calculator, and LogP calculator (Version 9). LogP was calculated for each whole molecule, and percent distribution of species and pKa values were calculated considering the hydrophobic component of the molecule containing the primary amine group (Murata and Yasumoto, 2000). The full molecule could not be handled by the program due to the large number of ionizable sites, especially on the hydrophilic portion of the molecule. Calculated values for logP (octanol:water partitioning coefficient when the compound is primarily unionized), logD (coefficient of octanol:water partitioning ratio of ionized to unionized over a pH range), and pKa (acid dissociation constant) are estimates based on the use of an extensive database of fragments and predicted

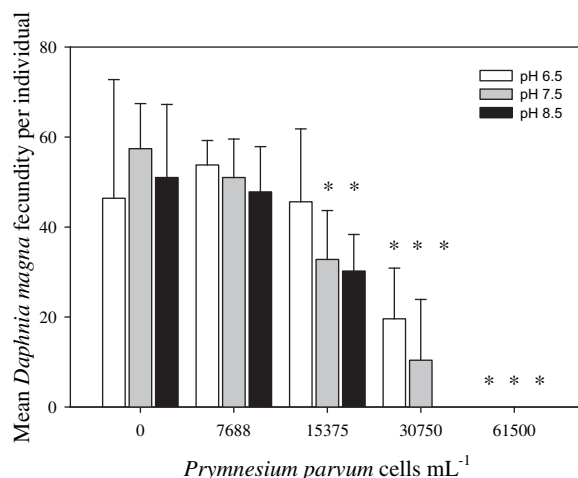


Fig. 2. Mean neonate production for *Daphnia magna* (\pm SD, $n = 5$) exposed to diluted samples of Lake Whitney water collected during a *Prymnesium parvum* bloom in 2007. Cell density is expressed as the % lake water multiplied by the density of *Prymnesium parvum* cells in the undiluted sample. The error bars represent standard deviations for each value. * represents treatments that are significantly lower than the respective controls ($p < 0.05$). Controls did not vary significantly among the three pH treatments ($p > 0.05$).

inductive effects based on substituents near ionizable sites. LogD was calculated as the sum of logD for the hydrophobic fragment at different pHs with the logP of the hydrophilic fragment (neutral form), which remains neutral at all relevant pHs (< 12).

3. Results

3.1. pH dependent toxicity in field studies: Lakes Granbury and Whitney

Ambient toxicity to juvenile *P. promelas* of Lake Whitney samples collected during a Spring 2007 bloom was reduced when pH was adjusted to < 7.5 (Fig. 1). The 48-h LC₅₀ value (95% confidence intervals) for experiments completed at pH 8.5 was 1.9×10^3 (1.6 – 2.7×10^3) cells mL⁻¹, whereas comparable values at pH 6.5 and 7.5 were 7.8×10^3 (7.1 – 8.8×10^3) and 4.1×10^3 (2.6 – 5.3×10^3) cells mL⁻¹.

Table 1

The percent survivorship in undiluted samples and 48-h LC₅₀ values in terms of percent reservoir water for *Pimephales promelas* exposed to Lake Granbury samples from three stations during a *Prymnesium parvum* bloom in March 2007.

Site	Cells per ml	pH	% Survivorship in undiluted sample	48 h LC ₅₀ (% lake water)	Upper and lower 95% confidence intervals	48 h LC ₅₀ (<i>Prymnesium parvum</i> cell/ml)
1	29×10^3	6.5	81	$> 100^a$	nc	nc
		7.5	71	$> 100^a$	nc	nc
		8.5	24	72	61–85	21×10^3
2	36×10^3	6.5	38	80	59–100	28×10^3
		7.5	5	54	41–71	19×10^3
		8.5	0	22	15–31	78×10^2
3	36×10^3	6.5	90	$> 100^a$	nc	nc
		7.5	67	$> 100^a$	nc	nc
		8.5	0	43	36–53	16×10^3

nc = Not calculable.

^a = There was insufficient mortality to generate a point estimate.

Table 2

The 48- and 96-hr LC₅₀ values in terms of percent reservoir water for *Daphnia magna* exposed to a composite sample obtained from Lake Granbury during a *Prymnesium parvum* bloom in 2007.

Time	pH	LC ₅₀ (% site water)	Upper and lower 95% confidence intervals	LC ₅₀ (<i>Prymnesium parvum</i> cell/ml)
48	6.5	>100 ^a	nc	34 × 10 ³
	7.5	65.5	42–100	22 × 10 ³
	8.5	46.7	31–70	16 × 10 ³
96	6.5	>100 ^a	nc	34 × 10 ³
	7.5	57.4	47–70	19 × 10 ³
	8.5	30.7	26–37	10 × 10 ³

nc = Not calculable.

^a = There was insufficient mortality to generate a point estimate.

There was a similar pH-dependent toxicological relationship during experiments with *D. magna*, as fecundity was significantly reduced at lower densities of *P. parvum* cells when exposure occurred at higher pH (Fig. 2). No reproduction was observed at any pH in 100% Lake Whitney water. There was no significant difference in reproduction between controls at each pH or unmodified RHW.

Susceptibility of *P. promelas* to samples from Lake Granbury during the 2007 bloom also indicated a pH-dependent toxicological relationship. Ambient toxicity to fish was ameliorated in lake samples from two stations and substantially reduced in a third by lowering pH (Table 1). There was insufficient mortality for samples collected from two sites to calculate LC₅₀ values at pH 6.5 and 7.5; thus, these values are conservatively reported as >100%. The sample from Site 2 was the only Lake Granbury sample for which LC₅₀ values could be determined for pH 6.5, 7.5, and 8.5. There was approximately a four-fold difference in LC₅₀ values between the pH 6.5 and 8.5 treatments, with higher toxicity at higher pH (Table 1). Similarly, toxicity to *D. magna* was reduced in low pH in a 96-h experiment exposing individuals to a composite sample from all three sites (Table 2). LC₅₀ values could not be calculated at pH 6.5 due to insufficient mortality, but point estimates for pH 7.5 and 8.5 differed by nearly two-fold, with higher toxicity to *D. magna* at higher pH (Table 2).

3.2. pH dependent toxicity in laboratory cultures

Cultures were terminated on day 28 after reaching late stationary phase, and experiments were immediately performed to assess the toxicity of each replicate culture. Cells were enumerated at this time showing densities in high nutrient (f/2) cultures of 2.0×10^5 , 1.5×10^5 , and 2.1×10^5 cells mL⁻¹, and densities in low nutrient (f/8) cultures of 1.5×10^5 , 1.3×10^5 , and 1.5×10^5 cells mL⁻¹. Survival in the ASW and RHW controls was >90% for all tests at all pH levels. The LC₅₀ values for experiments with *P. promelas* were consistently lower for the low nutrient (f/8) treatment compared to those for high nutrient (f/2) (Fig. 3). Estimated LC₅₀s were more variable between replicates for the f/2 treatment and increased exposure time resulted in greater toxicity, whereas temporal effects were less evident for the f/8 treatment (Fig. 3).

Samples of f/2 and f/8 whole cultures and cell-free filtrate were consistently more toxic to *P. promelas* when exposure occurred at pH 8.5 compared to pH 7.5 or 6.5

(Fig. 4). For the f/2 treatment, 50% of exposed individuals died at pH 6.5 in undiluted whole culture; however, only 15% died in the cell free filtrate. Cell free filtrates were also less potent than the whole culture at pH 7.5 and 8.5 for the f/2 treatment; however, differences in toxicity between whole cultures and cell-free filtrates were not as apparent for the f/8 treatment (Fig. 4). The LC₅₀ values were markedly lower for filtered and unfiltered cultures grown in f/8 media compared to those in f/2 media; however, endpoints were consistently lower at higher pH for all experiments (Table 3).

3.3. Prymnesin-1 and -2 physicochemical properties

The structures of prymnesin-1 and -2 (Fig. 5) lead to an estimated pK_a value of 8.9 for both prymnesin-1 and -2 (Table 4). LogD between pH 6.5 and 8.5 ranged between 3.4 and 5.2 for prymnesin-1, and 2.5 and 4.9 for prymnesin-2, respectively. At pH 6.5 approximately 16% of the prymnesins are predicted to be ionized, whereas at pH 8.5 only 0.002% are predicted to be ionized (Table 4).

4. Discussion

Our studies with laboratory cultures and samples from reservoirs experiencing *P. parvum* blooms consistently indicate that toxins released by *P. parvum* are more potent

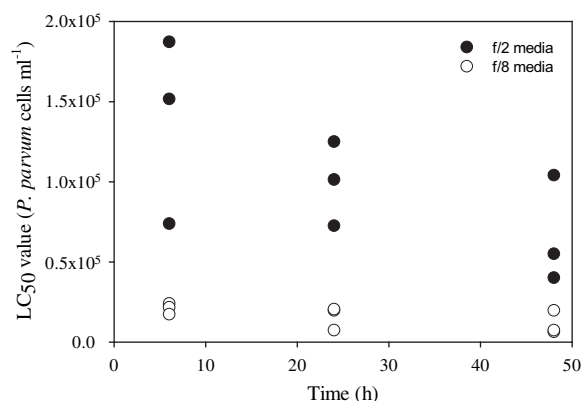


Fig. 3. LC₅₀ values for *Pimephales promelas* exposed to cultures of *Prymnesium parvum* grown in the laboratory using two different nutrient conditions (high nutrients – f/2 medium; low nutrients – f/8 medium). Each data point represents the LC₅₀ value for an individual culture.

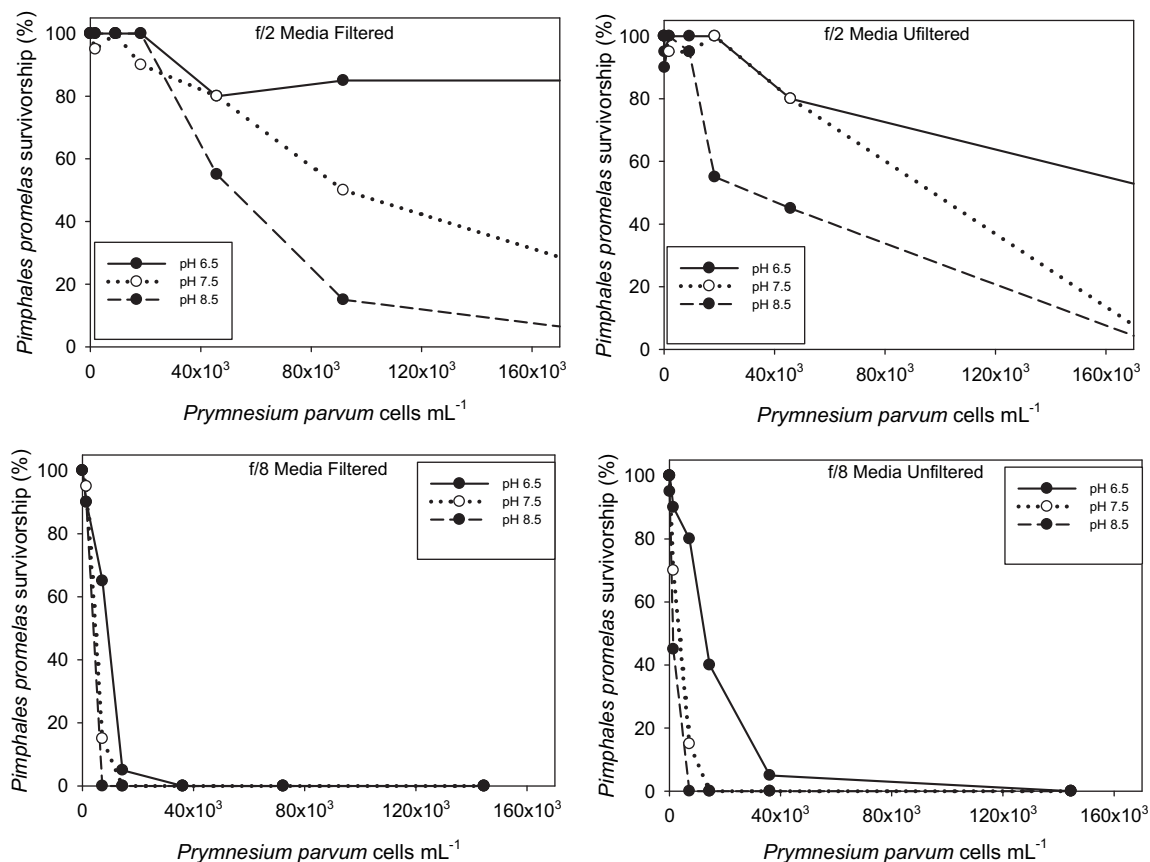


Fig. 4. The percent survivorship of *Pimephales promelas* exposed to samples of *Pymnesium parvum* grown under different nutrient conditions with cells (whole culture) and cells removed (filtrate).

when exposure occurred at a higher pH of 8.5 than at lower pH. The predicted physicochemical properties of prymnesins indicate that these toxins are weak bases ($pK_a = 8.9$) and, thus, a greater proportion of the prymnesins were likely unionized in higher pH treatment levels (e.g., 8.5). We propose that a higher proportion of prymnesins in unionized forms at pH 8.5 explains the greater toxicity observed in field and laboratory studies. This novel explanation for pH-dependent ambient toxicity associated with *P. parvum* suggests that variability in pH among and within aquatic systems may be an important factor governing the occurrence of fish kills.

Unionized forms of contaminants often have greater propensity to cross cellular membranes due to their lower polarity and thus are more likely to partition into organisms (Simon and Beevers, 1951; Sarrikoski et al., 1986; US EPA, 1986; Fisher et al., 1999; US EPA, 1999; Nakamura et al., 2008; Valenti et al., 2009). The importance of ionization state for ambient toxicity and environmental management is evidenced by the integration of site-specific ambient water quality criteria for contaminants such as pentachlorophenol and ammonia (US EPA, 1986,1999). Ammonia, like the prymnesins, has a pK_a value of ~ 9 . In addition, ammonia is a weak base and the ionization state of the compound changes appreciably across environmentally relevant surface water pH gradients (US EPA, 1999).

Consequently, acceptable ammonia loads in stream are 13-fold lower if the receiving system has a pH of 9 compared to a pH 6. Weak bases have a greater propensity to cross cellular membranes if the pH at which the

Table 3

The LC_{50} value and respective 95% confidence intervals for experiments completed with *Pimephales promelas* and cultures of *Pymnesium parvum* grown in f/2 and f/8 media that were either unfiltered or filtered to remove cells.

Media	Treatment	pH	LC_{50} value (% media)	Upper and lower 95% confidence intervals
f/2	Unfiltered	6.5	>100 ^a	nc
		7.5	35	26–46
		8.5	18	12–25
f/2	Filtered	6.5	>100 ^a	nc
		7.5	51	36–73
		8.5	30	23–40
f/8	Unfiltered	6.5	7	5–10
		7.5	1.7	1.4–2.4
		8.5	0.7	0.4–1.1
f/8	Filtered	6.5	4.3	3–6
		7.5	2.6	1.8–3.6
		8.5	2	1.1–2.7

nc = Not calculable.

^a = There was insufficient mortality to generate a point estimate.

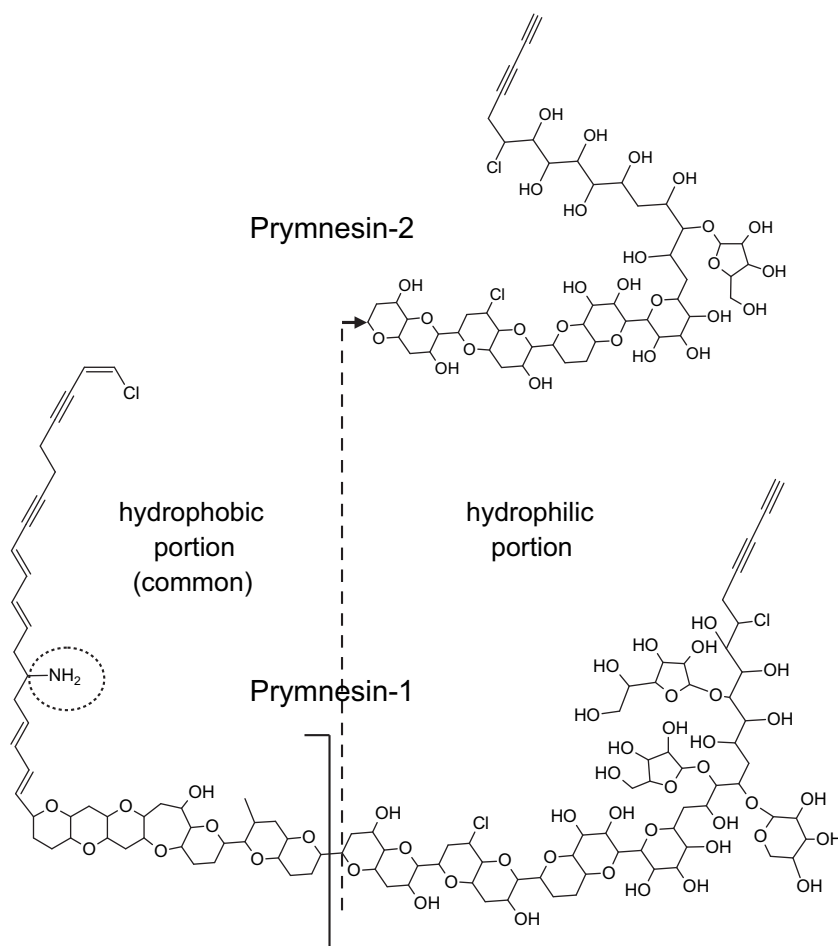


Fig. 5. The structures of prymnesin-1 and prymnesin-2 with the hydrophobic and hydrophilic portions of each compound differentiated. The primary amine is highlighted.

exposure occurs approaches and surpasses the compound's pKa value (US EPA, 1999, 1986; Simon and Beevers, 1951; Nakamura et al., 2008; Fisher et al., 1999). Greater interaction with target sites (e.g., gill membranes) would increase the likelihood of adverse effects in exposed individuals; hence, prymnesin-1 and -2 would pose greater risk to aquatic life when these toxins exist predominantly as the unionized form.

In laboratory tests examining the effectiveness of ammonium and barley straw extract to control *P. parvum*, Grover et al. (2007) only observed toxicity in samples with pH > 8. These observations were consistent with Lindholm et al. (1999) who observed fish kills attributed to *P. parvum* in a brackish-water lake when pH ranged between 8.9 and 9.4. Additional studies with *P. parvum* conducted under higher salinity conditions have reported similar pH influences during *in vivo* experiments. Shilo and Aschner (1953) noted that fish were 5-times more sensitive at pH 9 compared to pH 6, and demonstrated that the effects of pH manipulation were reversible during adjustments to and from pH 7 and 6. Ulitzur and Shilo (1964) investigated the toxicity of *P. parvum* toxins, along with various chemicals identified as cofactors, over a range of pH 7–9 and

consistently noted markedly greater toxicity at higher pH. McLaughlin (1958) observed that high pH shortened the exposure time associated with onset of mortality. The pH-dependent activity of *P. parvum* toxins could also reduce internal damage to cells that are producing or storing toxins. Extracts of *P. parvum* induced "self-toxicity," reducing growth rates and causing lysis (Olli and Trunov, 2007). It is plausible that these ionizable toxins are stored

Table 4

The predicted physicochemical properties of prymnesin-1 and -2 based on computer modeling and hand computation.

Property	Prymnesin-1	Prymnesin-2
Log <i>P</i>	6.0 ± 1.5	5.6 ± 1.5
Log <i>P</i> (hydrophobic portion)	7.5 ± 0.8	7.5 ± 0.8
Log <i>P</i> (hydrophilic portion)	−1.7 ± 1.5	−2.0 ± 1.5
Log <i>D</i> (pH 6.5)	3.4 ± 1.5	3.1 ± 1.5
Log <i>D</i> (pH 7.5)	4.3 ± 1.5	4.0 ± 1.5
Log <i>D</i> (pH 8.5)	5.2 ± 1.5	4.9 ± 1.5
% ionized (NH ₃ ⁺) (pH 6.5)	16	16
% ionized (NH ₃ ⁺) (pH 7.5)	0.02	0.02
% ionized (NH ₃ ⁺) (pH 8.5)	0.002	0.002
pKa (1° amine)	8.9 ± 0.1	8.9 ± 0.1
pKa (hydroxyl groups)	13–15	13–15

inside cells of *P. parvum* at lower physiological pH, and are thus more ionized than when released outside the cell, where pH values may be higher.

Some previous results from *in vitro* hemolytic experiments with *P. parvum* contradict the results of the *in vivo* experiments reported here and elsewhere. Blood cells rupture more often when exposures are completed at pH < 6 (Igarashi et al., 1996; 1998; Kim and Padilla, 1977). Pymnesin-1 and -2 have multiple ionizable groups so that changes in the protonation state could alter the configuration of the toxins. In turn, the interaction of pymnesins with specific binding sites in blood cells and fish gill membranes could depend on the structural configuration, which may be influenced by pH, and thus may be different among such *in vivo* and *in vitro* experiments. Alternatively, pymnesins might not be the only, or even the most important toxins produced by *P. parvum*, and hemolytic activity *in vitro* might have different determinants than lethal activity *in vivo* (Schug et al., in press).

Cell density alone has been long recognized as a poor predictor of toxicity for samples containing *P. parvum* (Reich and Aschner, 1947; Baker et al., 2007; Grover et al., 2007), and this generalization remains apparent during monitoring in Texas reservoirs. Ionization state of the toxins may partially explain some of this variability and reduce uncertainty related to ecological risk assessments and risk management of *P. parvum* blooms. The observed pH-dependent toxicological relationships and the physicochemical properties predicted by computer modeling suggest that the toxins pymnesin-1 and -2 act as weak bases in aqueous solutions. Because their predicted pKa values are within the range of variation of pH in many surface waters, modest variations in pH could have a large influence on toxicity.

The production of ionizable toxins offers potential advantages to *P. parvum* and may be related to biochemical adaptations associated with its marine origins. The results of our studies and others suggest that the toxins released by *P. parvum* are more potent to gill-breathing organisms when exposure occurs at pH levels representative of those measured in marine systems (e.g. pH > 8). Moreover, blooms of *P. parvum* and other HABs can alter the environment and cause pH to increase through depletion of carbon dioxide during daytime photosynthesis (Pearl, 1988). In fish hatchery ponds impacted by *P. parvum*, pH measurements vary by more than one unit between the daylight and evening hours (Shilo and Shilo, 1953). Thus, *P. parvum* not only produces toxins during bloom formation, but could also make conditions that increase the potency of their toxins. For example, our research team recently observed high pH levels in Lake Granbury during a *P. parvum* bloom that resulted in ambient toxicity to fish, compared to lower pH levels before and after this bloom (Roelke et al. in review).

Considering site-specific pH may be especially important for ecological risk assessments of *P. parvum* because of the inherent linkage between physicochemical properties of waters and the organisms that inhabit them. There is far greater spatiotemporal variability in the pH of inland waters compared to marine systems. Some of this variability arises from natural variations in geomorphology,

geochemistry, and climate. Anthropogenic activities also influence the pH of inland waters. Inland waters where *P. parvum* blooms have occurred are often affected by altered hydrology, land use changes in the catchment, and increased nutrient loading. In the southwestern and south central U.S., *P. parvum* blooms and fish kills are often limited to waters where pH is typically high due to an arid climate, limestone bedrock, and sparse vegetation. Consequently, prospective ecological risk assessment approaches may be possible for predicting the occurrence of harmful blooms of *P. parvum* by relating watershed land-use and geography to water quality.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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